University of California Office of the President
Special Research Programs

Annual Progress Report
SUMMARY OF SCIENTIFIC PROGRESS
Form 3

(Check one) [X] Breast Cancer Research [ ] Tobacco-Related Disease Research [ ] Universitywide AIDS Research

AWARD NUMBER: 7KB-0017 PROJECT YEAR *(Check one)*: [ ] 1st [ ] 2nd [ ] 3rd [X] Final

PRINCIPAL INVESTIGATOR(S): John L. Muschler, Ph.D.

PROJECT TITLE: Tumor Suppression by Dystroglycan in Breast Epithelial Cells

INSTITUTION: E.O. Lawrence Berkeley National Laboratory

The summary must include:

a) A statement of each specific aim, followed by an account of progress made towards its accomplishment, including a summary of experimental results.
b) If an original aim was dropped or modified, an explanation of the reason for such a change.
c) If a new aim was added, progress toward its achievement.
d) The relevance of any modified or new specific aim(s) to the project's mission and research priorities

Do not exceed 5 pages; number any additional pages as 3a, 3b, etc.

(Collaborative Grants should submit one combined progress report.)

(See Sections 7.3 and 7.4 for special TRDRP and UARP reporting requirements.)

Dystroglycan is a laminin receptor expressed in many tissues including breast epithelial cells [1]. In previous work, I had implicated DG in mediating the normal response of cells to the basement membrane, and also found that DG function to be compromised in many tumor cell lines. I have restored dystroglycan (DG) activity to a tumorigenic cell line (T4 cells) through cDNA overexpression. Restoration of DG function in this cell line re-instated normal responses to basement membrane proteins, including cell polarization, growth arrest and cytoskeletal changes. Restoration of DG function also eliminated the tumorigenic potential of T4 cells in nude mice, implicating DG as a tumor suppressor.

Specific Aim I: Determine the downstream effects of DG signaling through conditional activation in mammary tumor cells and conditional knockout in primary cultures.

Downstream signaling events from DG are being assayed in control cells and in tumor cells wherein DG function was restored by cDNA over-expression. Initial targets have included the Rho family of GTPases. In T4 cells, activated Rac was easily measurable, and levels were determined to be higher in the tumor cells than in non-tumorigenic counterparts. As yet, activated Rho and Cdc42 have not been observed at detectable levels in T4 cells. T4 cells +/- DG activity are currently being tested to determine if Rac activity is modulated by DG activity.

Previous work had detected an up-regulation of PTEN in DG over-expressing tumor cells grown within a 3-D gel of basement membrane proteins (Matrigel). I have asked if PTEN up-regulation was a direct or indirect effect of DG signaling, and whether up-regulated PTEN activity could also explain the observed "cell rounding" in response to laminin; cell rounding is mediated by DG and occurs when cells cultured on plastic are exposed to Matrigel. PTEN up-regulation did not accompany cell rounding, therefore, PTEN is not thought to be an initial mediator of DG signals, but is indirectly regulated by other DG signaling events such as cell polarization or growth arrest.

DG over expression was previously found to restore polarity to the T4-2 cells in defined medium which did not contain EGF. Addition of EGF to the medium was found to disrupt polarity in the DG over-expressing cells, effectively over-riding DG function. Therefore, EGF signaling was found to compete downstream with signals from DG. Addition of the MEK-1 inhibitor again restored polarity. These results now place focus on events downstream of MAPK as antagonists of DG function.

Invasion assays were performed using standard Matrigel coated membranes and assaying cell migration through the membranes. Control T4 cells were moderately invasive in these assays, and DG over-expression did not change their invasive potential. This was surprising, and demonstrated that the migration of these cells through Matrigel was not regulated by DG activity.

The tool for creation of the conditional DG knockout is a transgenic mouse line wherein the DG gene is flanked by "lox" site (called the "floxed-DG" mouse line). This mouse line was developed in the laboratory of Dr. Kevin Campbell. Arrival of the floxed-DG mouse line in my laboratory was delayed for reasons of quality control, and did not come into my possession until September of 2001. The transgenic mice were first breed and expanded so that homozygous (fl/fl) animals became available for experimentation. Others are being back-crossed into a background of C57/BL6, so that tissues can be transplanted into the cleared fat pad of wild-type mice without complications from strain variation. More than 30 homozygous females have been obtained from the original mixed strain. Primary mammary epithelial cultures have been obtained from 14 of these homozygous mice. The first set of primary cultures was treated for the establishment of immortalized cell lines possessing two copies of the floxed-DG locus. We have attempted spontaneous immortalization and also immortalization by infection with the E7 antigen. These efforts are continuing at present, and appear successful. Additional primary cultures were stocked in the freezer, ready for manipulation, using a "Cre"-expressing adenovirus.

Cre-expressing adenoviruses have been acquired that express the transgene from two different promoters. We have created frozen stocks of each virus, and are testing each for there efficiency in transgenesis, and deletion of the DG gene. Each is also being tested for unwanted effects of cre over-expression in wild-type mammary epithelial cells. Creation and analysis of DG-/- cells is scheduled to begin in the next month.

Specific Aim II: Determine, by site-directed mutagenesis, which signaling pathways emanating from DG are critical for tumor suppression.

These experiments are awaiting the creation of DG-/- cells from the "floxed-DG" transgenic mouse line. Scheduled for the Spring of 2002.

Specific Aim III: Determine how the critical DG signaling pathways are compromised in tumor cells, in order to find methods to restore DG signaling functions.

This aim has seen the most progress. In an effort to understand why DG function was compromised in many tumor cell lines, I analyzed the expression and molecular characteristics of DG in multiple human breast tumor cell lines. In a set of human breast tumor cell lines, I assayed by immunoblot the relative levels of α and β-DG, β1 integrins and E-cadherin (Figure 1A). Strikingly, α-DG levels were highly variable among the different cell lines. Only three of the nine tumor cell lines (BT474, Skbr-3 and T47D) showed the presence of high levels of α-DG, while the others displayed relatively minor levels of the protein. This same pattern of α-DG protein levels was observed when

probing these samples using a polyclonal α-DG antibody and using a laminin-overlay assay [3](not shown).

Significantly, β-DG was displayed in a very distinct pattern from that of α-DG. β-DG levels were prominent in all of the cell lines except T4-2s, with relatively less variation among the cell lines than observed for α-DG. Both DG subunits, α and β, are the product of a single gene, cleaved into two subunits by post-translational processing [4]. Therefore, it could be expected that the ratio of α- to β-DG protein would be constant in all cell lines. However, among the cell lines tested here, BT474's showed the highest α-DG to β-DG ratio, with all other cell lines displaying a lower ratio. This indicated that some quantity of α-DG was lost from the cell surface in most, if not all cell lines tested, and the lack of α-DG in most tumor cells resulted from a high degree of α-DG "shedding". The one exception was T4-2 cells, which displayed lower expression of both α and β-DG and a lesser degree of α-DG shedding.

α-DG is required for substrate binding and is therefore required for receptor function. Seeing the widely variable levels of α-DG protein among the 9 cell lines in figure 1, we next asked whether the relative levels of α-DG predicted the ability of these cells to respond correctly to BM proteins. Functionally normal cells cultured within a 3-dimensional gel of reconstituted BM proteins (Matrigel) grow from single cells to form polarized multi-cellular epithelial structures, whereas most tumorigenic mammary epithelial cells grow as disorganized cell masses [5]. This test is referred to here as the "3D BM assay".

Each of the tumor cell lines analyzed in Figure 1, was cultured within the 3D BM assay. The resulting structures were subsequently examined for the formation of a polarized epithelial cell layer (Figure 2). Polarity was defined by basal α6 integrin localization, apical localization of the Golgi, and basal localization of nuclei. Among the nine cell lines tested, only two cell lines were observed to form polarized structures when cultured in the 3D BM assay; T47D and BT474 each showed basal polarization of nuclei and α6 integrin, and apical polarization of the Golgi (Figure 2c and d and not shown). T47D cells formed large colonies with clearly polarized cells contacting the BM proteins. The BT474 cells, having the highest surface levels of α-DG, were more growth restricted in the 3D-BM assay; even then, colonies of 4 cells or more were polarized. T4-2 cells, MDA-MB-468 cells, and MDA-MB-453 cells were apolar, as determined by α6 integrin localization and random nuclei distribution (Figure 2a, b, c). MCF-7 and Skbr-3 cells did not display detectable α6 integrin staining, but were judged apolar by random Golgi and nuclei distribution (Figure 2c and Figure 3a). MDA-MB-231 and MDA-MB-435 cells were apolar (not shown) and also displayed invasive behavior within the Matrigel (Figure 2g and h), consistent with their highly invasive behavior in vivo [6].

Among the cells in figure 2, those that possessed the ability to polarize in response to the BM (BT474 and T47D cells) each displayed relatively high levels of α-DG, while those that lacked the ability to polarize displayed low α-DG levels. Therefore, the levels of α-DG accurately predicted the behavior of these eight cell lines. The one exception was Skbr-3 cells, which failed to polarize (Figure 3a), despite displaying relatively high levels of α-DG. These cells instead grew as loose groups of individual cells with minimal cell-cell interaction. We hypothesized that the inability of Skbr-3 cells to polarize resulted not from an inability to sense the BM, but instead from a lack of cell-cell adhesions, since Skbr-3 cells notably lacked E-cadherin expression (Figure 1A) [7]. This prediction was tested by re-expressing the E-cadherin cDNA in Skbr-3 cells by retroviral infection and selection for the infected cell population. As

seen in figure 3, the population Skbr-3 cells expressing E-cadherin regained the ability to form polarized structures. Polarity was observed in greater than 80% of Skbr-3 cells infected with the E-cadherin expressing virus, compared to no polarized colonies observed in cells infected within an empty virus as control. In contrast, expression of E-cadherin in MDA-MB-231 cells, which possessed relatively low α-DG levels, did not permit polarization of any cells in the population (not shown).

The results presented above established that α-DG levels can predict cell responses to the basement membrane, and reinforced the model that α-DG plays a role in the ability of cells to form a polarized epithelial cell layer in response to the BM. This model predicts that augmenting the levels of α-DG would alter the response to BM proteins in those cells where α-DG levels are insufficient to effect correct responsiveness. Over-expression of the DG cDNA, by cell transfection, would be a straight-forward method to augment the levels of α-DG in a tumor cell, if α-DG shedding were not a dominant factor.

To test the ability of DG over-expression to alter α-DG levels in these tumor cells, we introduced the DG cDNA in those cell lines that failed to polarize in the presence of BM. Cells of each cell line were infected with a retrovirus expressing the full-length human DG cDNA, or infected with an empty vector as a control. After infection, cells were selected for neomycin resistance (also encoded by the virus) and populations of infected cells were obtained. Immunoblots of both α- and β-DG from the infected cells showed that while β-DG levels were elevated in each cell line over-expressing the DG cDNA, α-DG levels were elevated in only two of the cells lines: Skbr-3 and HMT-3522-T4-2 (not shown). This result was not surprising in light of the data presented in figure 1, showing the relative degree of α-DG shedding in each these cell lines. Over-expression of the DG cDNA was not sufficient to over-ride the high degree of α-DG shedding, which appears to be the dominant regulator of α-DG levels in MCF-7, MB-MB-468 cells, MDA-MB-231, MDA-MB-453, and MDA-MB-435 cells. In contrast, shedding of α-DG was relatively minor in Skbr-3 and T4-2 cells, therefore, over-expression of the DG cDNA resulted in proportionally higher levels of α-DG in these cells. Significantly, this result also confirms that loss of α-DG from the cell surface <u>does not results from genetic alterations in the DG gene.</u>

Cell populations over-expressing DG were tested for their ability to polarize in the 3D-BM assay. As expected, over-expression of the DG cDNA did not change the behavior of those cells that displayed dominant α-DG shedding (not shown).

As I have previously demonstrated, elevation of α-DG proteins levels in T4-2 cells restored the ability of the cells to form polarized epithelial structures when grown within the 3D BM assay. Polarity was observed by basal α6 integrin localization, by polarized deposition of BM proteins (laminin-5), and by apical polarization of the Golgi apparatus.

Finally, (prior to activation of BCRP funding) I have demonstrated that shedding of α-DG can be caused by proteolysis. The α-DG molecule is detected in the supernatant of some tumor cells, but is smaller than the molecule detected at the cell surface (Figure 6). Shedding of α-DG into the medium is inhibited by the hydroxamate metalloproteinase (MP) inhibitor GM6001 at a Ki of ~10 μM. Therefore, there is good evidence that α-DG is shed, at least in part, by the activity of a metalloproteinase. This also suggests that MP inhibitoris can be used as a tool to restore DG function in tumor cells. Indeed, treatment of some tumor cell lines with MP inhibitors restored their ability to polarize in the 3D-BM assay. Under funding of the CA-BCRP I have tested a panel of know MP inhibitors in the

3D-BM assay to determine those most effective. Although most were effective, none were effective below a 500 nM concentration, in contrast to the majority of MPs, which are generally inhibited at concentrations around 1nM. This indicates that the critical MP in this study is particularly insensitive MP inhibitors and may reside in a class of MPs that is distinct from the the common matrix MPs. The challenge now is to identify the MP cleaving DG, and to demonstrate the consequences of this one cleavage event to breast epithelial cell behavior.

References

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